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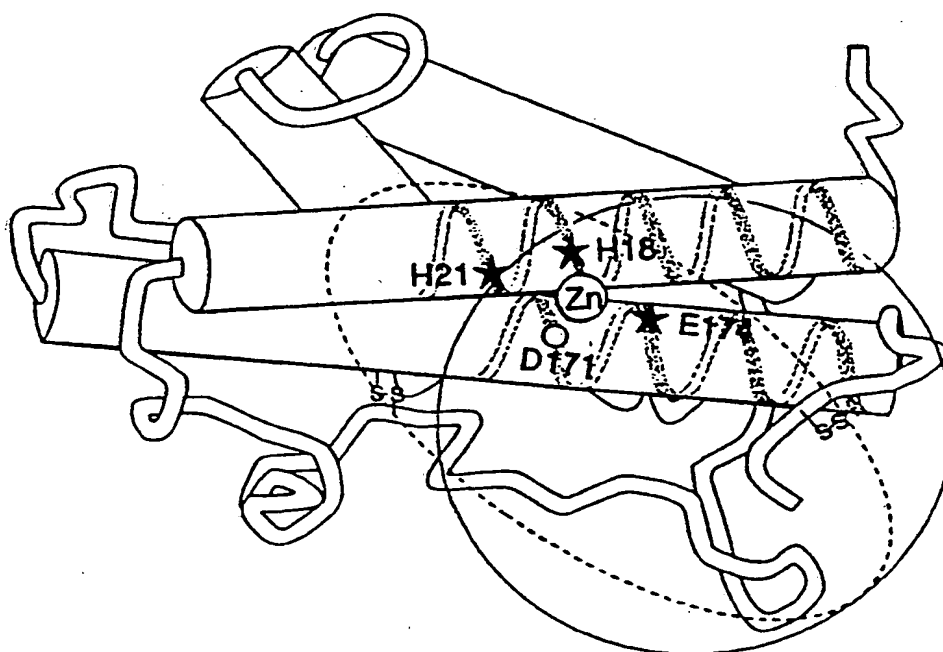
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(54) Title: STABLE GROWTH HORMONE METAL ION FORMULATIONS



(57) Abstract

Described are stable divalent metal ion-human growth hormone (hGH) complexes resulting in a stabilized hGH formulations through the formation of hGH-metal ion dimers. The stable dimers are characterized and therapeutic formulations of zinc and hGH are described.

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## STABLE GROWTH HORMONE METAL ION FORMULATIONS

BACKGROUND OF THE INVENTION5 Field of the Invention

Stable formulations of human growth hormone (hGH) are described which incorporate a divalent metal ion, such as zinc and hGH. The use of such hGH formulations having improved stability and therapeutic efficacy are described.

Description of the Background Art

1 0 Human growth hormone (hGH) is synthesized and secreted into storage granules prior to its release from the anterior pituitary (for review see W. F. Daughaday, in *Textbook of Endocrinology*, seventh edition, Chapter 18, ed. J.D. Wilson and D.W. Foster, (W. B. Saunders Co., Philadelphia, 1985), p. 577; U.J. Lewis, *Ann. Rev. Physiol.* **46**, 33-42 (1984)). Histochemical analysis of the anterior pituitary indicates that zinc is present in high concentrations in growth hormone secretory granules (O. Thorlacius-Ussing, *Neuroendocrinol.* **45**, 233-242 (1987)).

1 5 Independently, it has been suggested that zinc may modulate release of growth hormone (GH) from the pituitary because high physiological concentrations of  $Zn^{2+}$  inhibit GH release (F. La Bella, R. Dular, S. Vivian, G. Queen, *Biochem. Biophys. Res. Commun.* **52**, 786-791 (1973); A.W. Root, G. Duckett, M. Sweetland, E.O. Reiter, *J. Nutr.* **109**, 958-964 (1979); M.Y. Lorensen, D.L. Robson, L.S. Jacobs *J. Biol. Chem.* **258**, 8618-8622 (1983)). However, the biochemical and structural bases whereby  $Zn^{2+}$  could be involved in the storage or release of hGH *in vivo* has not been elucidated. Zinc involvement in hGH binding to receptors was described in PCT Pub. WO92/03478, published 5 March 1992. The problems associated with inactive dimer, and non-zinc containing dimer formation in purified hGH that results in inactive hGH, were noted in  
2 0 Becker, G.W., *Biotechnology and Applied Biochemistry* **9**, 478 (1987).

2 5 Metal ions such as zinc have been shown to be useful in the prolonged parenteral release of somatotropins in an oil formulation (EP 177,478, published 04.10.84; EP 343,696, published 29.11.89). Similar slow release formulations of bovine growth hormone complexed with metal ion in an oil vehicle have been shown (EP 216,485, published 01.04.87). Metal ions have been used to  
3 0 recover somatotropin from dilute aqueous solutions by forming a precipitate (EP 277,043, published 03.08.88). Prolactin has been examined as a regulatory hormone for zinc uptake by the prostate gland (Leake *et al.*, *J. of Endocrinology* **102**(1), p73-76, 1984). Zinc deficiency has been associated with a tendency to hyperprolactinemia (Koppelman, *Medical Hypotheses*, **25**(2), p65-68, 1988). A review of the zinc requirement in humans can be found in Prasad, *Special Topics in Endocrinology and Metabolism*, vol 7, p45-76, 1985. Zinc somatostatin complexes have  
3 5 been shown to inhibit growth hormone release (U. S. Patents 3,998,795 and 3,926,937).

Human growth hormone (hGH) participates in much of the regulation of normal human growth and development. This 22,000 dalton pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like

and diabetogenic effects among others (Chawla, R. K. (1983) Ann. Rev. Med. **34**, 519; Edwards, C. K. et al. (1988) Science **239**, 769; Thomer, M. O., et al. (1988) J. Clin. Invest. **81**, 745). Growth hormone deficiency in children leads to dwarfism which has been successfully treated for more than a decade by exogenous administration of hGH.

5 hGH is a member of a family of homologous hormones that include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nicoll, C. S., et al., (1986) Endocrine Reviews **7**, 169). hGH is unusual among these in that it exhibits broad species specificity and binds to either the cloned somatogenic (Leung, D. W., et al., [1987] Nature **330**, 537) or prolactin receptor (Boutin, J. M., et al., [1988] Ce: **53**, 69). The cloned gene for hGH has  
10 been expressed in a secreted form in Escherichia coli (Chang, C. N., et al., [1987] Gene **55**, 189) and its DNA and amino acid sequence has been reported (Goeddel, et al., [1979] Nature **281**, 544; Gray, et al., [1985] Gene **39**, 247). The three-dimensional structure of hGH is not available. However, the three-dimensional folding pattern for porcine growth hormone (pGH) has been reported at moderate resolution and refinement (Abdel-Meguid, S. S., et al., [1987] Proc. Natl.  
15 Acad. Sci. USA **84**, 6434). Human growth hormone's receptor and antibody epitopes have been identified by homolog-scanning mutagenesis (Cunningham et al., Science **243**: 1330, 1989). The structure of novel amino terminal methionyl bovine growth hormone containing a spliced-in sequence of human growth hormone including histidine18 and histidine 21 has been shown (U.S. Patent 4,880,910)

20 Human growth hormone (hGH) causes a variety of physiological and metabolic effects in various animal models including linear bone growth, lactation, activation of macrophages, insulin-like and diabetogenic effects and others (R. K. Chawla et al., Annu. Rev. Med. **34**, 519 (1983); O. G. P. Isaksson et al., Annu. Rev. Physiol. **47**, 483 (1985); C. K. Edwards et al., Science **239**, 769 (1988); M. O. Thomer and M. L. Vance, J. Clin. Invest. **82**, 745 (1988); J. P. Hughes and H. G. Friesen, Ann. Rev. Physiol. **47**, 469 (1985)). These biological effects derive from the interaction between hGH and specific cellular receptors. Only two different human receptors have been cloned, the hGH liver receptor (D. W. Leung et al., Nature **330**, 537 (1987)) and the human prolactin receptor (J. M. Boutin et al., Mol. Endocrinol. **3**, 1455 (1989)). However, there are likely to be others including the human placental lactogen receptor (M. Freemark, M. Comer, G. Komer, and S. Handwerker, Endocrinol. **120**, 1865 (1987)). These homologous  
30 receptors contain a glycosylated extracellular hormone binding domain, a single transmembrane domain and a cytoplasmic domain which differs considerably in sequence and size. One or more receptors are assumed to play a determining role in the physiological response to polypeptide hormones.

35 A major biological effect of hGH is to promote growth in young mammals and maintenance of tissues in older mammals. The organ systems affected include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys. Growth hormone exert its action through interaction with specific receptors on the target cell's membrane.

In order for hGH to be available to the public as a therapeutic pharmaceutical compound, formulations must be prepared. Such formulations must maintain activity for appropriate extended periods of time, they must be acceptable in their own right for easy and rapid administration, and they must be readily formulated. Human growth hormone has been formulated in a variety of ways as described in U.S. Pat. No. 5,096,885. In many cases pharmaceutical formulations are provided in frozen or in lyophilized form. In such a case, the composition must be thawed or reconstituted prior to use. The frozen or lyophilized form is often used to maintain biochemical integrity and the bioactivity of the medicinal agent contained in the compositions under a wide variety of storage conditions, as it is recognized by those skilled in the art, lyophilized preparations often maintain activity better than their liquid counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of suitable pharmaceutically acceptable diluent(s), such as sterile water for injection or sterile physiological saline solution, or an appropriate physiologically acceptable diluent.

Alternatively, a pharmaceutical formulation can be provided in liquid form appropriate for immediate use. Desirable is a liquid formulation which maintains its activity in long term storage. Current formulations of hGH lose activity due to the formation of inactive dimers and higher order aggregates during formulation processing as well as during storage and reconstitution. Other chemical changes, such as deamidation, oxidation and enzymatic hydrolysis may also occur upon storage.

Prior attempts to prevent the formation of inactive dimers of hGH have not succeeded. The problems associated with inactive dimers are noted in Becker, G.W., Biotechnology and Applied Biochemistry 9, 478 (1987).

It is an object of the present invention to prepare stable formulations of hGH, both lyophilized and liquid, that do not result in the formation of inactive complexes of hGH nor cause chemical changes that result in a loss of activity or receptor specificity. Another object of the present invention is the preparation of formulations of liquid hGH, stable at room temperature or body temperature, thereby facilitating administration. Still another object is to provide a formulation which can be aerosolized for pulmonary use, or used in a needleless jet injector for subcutaneous injection. Yet another object of the invention is to provide an hGH formulation with enhanced therapeutic characteristics, such as the more even systemic delivery of hGH throughout the body, as opposed to a partial preference for the area surrounding the site of administration. Other objects, features and characteristics of the present invention will become more apparent upon consideration of the following description and the appended claims.

#### SUMMARY OF THE INVENTION

The present invention is based upon the discovery that the inclusion of a divalent metal ion such as zinc, cobalt or copper, preferably zinc, into an hGH formulation results in the formation of stable zinc:hGH dimers that exhibit unexpected stability to denaturation and maintain the activity of hGH for long periods at temperatures up to and beyond 37°C. Additional components of the stabilizing formulation may be a buffer, an amino acid, a bulking

agent or a nonionic surfactant. Other helical cytokines may be formulated using divalent metal ions, such as zinc, cobalt and copper. Among those cytokines formulations stabilized by divalent metal ions are human prolactin and human placental lactogen. Optimum stability of helical cytokines is achieved when the molar ration of divalent metal ion to helical cytokine is 1:1.

## 5 Brief Description of the Drawings

Fig. 1. Equilibrium dialysis and Scatchard analysis for binding of  $^{65}\text{Zn}^{2+}$  to hGH. From these Scatchard values the concentrations of bound and free zinc were calculated and it was determined that zinc forms a 1:1 complex with hGH whereby a dimer containing two zinc ions and two hGH is produced. Error bars indicate standard deviations from triplicate determinations.

Fig. 2. Gel filtration chromatography of hGH in the presence (A) or absence (B) of  $\text{ZnCl}_2$ . Size standards (bovine  $\gamma$ -globulin, ovalbumin and myoglobin) were run separately to calibrate the column, and their peak locations are indicated in the standard curve at the top of the chromatogram. In the absence of zinc (B) the hGH is a monomer, while in the presence of zinc (A) a dimer is formed which partially dissociates resulting in an apparent molecular weight that is 1.85 times the monomeric hGH peak.

Fig. 3. Sedimentation equilibrium analysis of hGH in the presence (●) or absence (○) of 20  $\mu\text{M}$   $\text{ZnCl}_2$ . Panel A shows the concentration of hGH (mg/ml) versus radial distance. Panel B shows  $\ln[\text{hGH concentration}]$  vs  $r^2$  which gives a linear plot in the absence of  $\text{ZnCl}_2$  (○), as expected for a monomeric species. The plot is curvilinear in the presence of  $\text{ZnCl}_2$  (●) showing self association.

Fig. 4A. UV difference spectra for binding of  $\text{Zn}^{2+}$  (—) and  $\text{Co}^{2+}$  (----) to hGH. Both zinc and cobalt induce hGH dimer formation in the same manner.

Fig. 4B. Visible difference spectra for binding of increasing concentrations of  $\text{Co}^{2+}$  to hGH.

Titration of hGH with  $\text{Co}^{2+}$  produces a visible absorption spectrum with a maximum at 525 nanometers and molar absorptivity at  $260 \text{ cm}^{-1}\text{M}^{-1}$ . The wavelength maximum and molar absorptivity are characteristic of  $\text{Co}^{2+}$  in a tetrahedral environment coordinated by nitrogen and oxygen containing ligands. Visible difference spectra are calculated from the before mixing spectrum of the tandem cells (1.0 cm) containing on one side 23  $\mu\text{M}$  hGH and on the other side  $\text{Co}^{2+}$  ranging from 10 to 2500  $\mu\text{M}$ , and the spectrum after mixing the components.

Fig. 5. Dimerization of hGH (wild type) and hGH mutants by addition of zinc. Panels A-D show elution profiles for hGH and the designated hGH mutants. Mutants are indicated by the single-letter amino acid for the wild-type residue followed by its position in mature hGH and then the mutant residue. Within each panel are superimposed gel filtration chromatograms run in buffers containing a fixed concentration of either 10  $\mu\text{M}$   $\text{ZnCl}_2$  (shaded peaks) or 1 mM EDTA (unshaded peaks).

Fig. 6. Panel A. Folding model for hGH based upon a 2.8 Å structure of porcine GH (25) showing the putative location of the  $\text{Zn}^{2+}$  ligands (starred) and the extent of the prolactin receptor (dashed ellipse) and hGH receptor (solid circle).



Panel B. One of three possible bridged ligand models that can account for the involvement of His18, His21, and Glu174 in coordinating 2 zinc atoms per hGH dimer. A salient feature of this model is the two-fold symmetry axis through the center of the "twinned" metal site. Glu174 from each hormone is shown coordinating both  $Zn^{2+}$  atoms simultaneously. It is also possible that either His18 or His21 can bridge both zinc atoms instead of Glu174.

Fig. 7. Stability of hGH to denaturation by Gu-HCl in the presence (●) or absence (○), of 25  $\mu M$   $ZnCl_2$ . A solution containing 30  $\mu g$  hGH, 0.01 M Tris buffer pH 8.0, with or without 25  $\mu M$   $ZnCl_2$  and the indicated GuHCl concentration was equilibrated for 2 min at 25°C prior to measuring the  $\theta_{222}$  in a 1 cm CD cell. For those samples containing  $Zn^{2+}$ , the  $Zn^{2+}$  was added before the GuHCl. The fraction folded was calculated from the fractional change in  $\theta_{222}$  in the absence of Gu-HCl (folded) relative to that in 6.5 M Gu-HCl (unfolded).

#### Description of the Preferred Embodiments

Novel formulations of zinc and hGH result in a stable hGH formulation suitable for prolonged storage, and for therapeutic administration. Therapeutic formulations containing the  $Zn^{2+}$ -hGH dimer are extremely stable, even at body temperature, while still allowing therapeutic administration of the formulation. The presence of zinc induces hGH to dimerize cooperatively by coordinating the same set of ligands used to bind hGH to the extracellular domain of the human prolactin receptor (hPRLbp) (B. C. Cunningham, S. Bass, G. Fuh, J. A. Wells *Science* 250, 1709-1712 (1990)).

#### 20 Formulation of Divalent Metal Ion and hGH

The present invention is based upon the discovery that the inclusion of a divalent metal ion, preferably zinc, into an hGH formulation results in the formation of stable zinc:hGH dimers that exhibit unexpected stability to denaturation, maintain the activity of hGH for periods at temperatures between -76°C and 60°C, particularly for long periods about 37°C., and are not susceptible to undesirable reactions otherwise encountered during processing, reconstitution and storage. As used herein, the term processing includes filtration, filling into vials, and lyophilization.

Therapeutic formulations of an hGH:metal ion formulation for therapeutic administration are prepared for storage by mixing hGH and metal ion having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980)., in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or

sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween. Plurionics or polyethylene glycol (PEG).

The invention is thus directed to such formulations, and to all associated formulations and as a means for effectively stabilizing human growth hormone. The formulation contains at least a divalent metal ion selected from zinc, cobalt or copper and substantially pure hGH free of contaminating proteins or infectious agents found in humans. Formulations of the present invention may additionally contain a pharmaceutically acceptable buffer, amino acid, bulking agent and/or non-ionic surfactant. These include, for example, buffers, chelating agents, antioxidants, preservatives, cosolvents, and the like; specific examples of these could include, trimethylamine salts ("Tris buffer"), and disodium edetate.

#### hGH Compositions

As used herein, the terms "human growth hormone" or "hGH" denote human growth hormone produced, for example, from natural source extraction and purification, and by recombinant cell culture systems. The native sequence of hGH and its characteristics are set forth, for example, in Hormone Drugs, Gueriguigan *et al.*, U.S.P. Convention, Rockville, MD (1982). The terms likewise cover biologically active human growth hormone equivalents; e.g., differing in one or more amino acid(s) in the overall sequence. Further, the terms as used in this application are intended to cover substitution, deletion and insertion amino acid variants of hGH, or post translational modifications. Examples of such variants are described in PCT Pub. WO90/04788, published 3 May 1990. Another hGH modification is that created by the covalent addition of polyethylene glycol to reactive hGH amino acids (Davis *et al.* U.S. patent 4,179,337). The hGH used in the formulations of the present invention is generally produced by recombinant means as previously discussed. This formulation recombinant hGH is substantially pure, free of other human proteins, free of infectious agents such as the human immunodeficiency virus (HIV) and it is soluble, both as a monomer and as a metal ion containing dimer. "Substantially pure" hGH means hGH that is free of vesicular proteins with which it ordinarily is associated in pituitary cells. Ordinarily, substantially pure means hGH which is greater than about 95% pure by weight of total protein, and preferably greater than 98% pure by weight.

A "pharmaceutically effective amount" of hGH refers to that amount which provides therapeutic effect in various administration regimens. The compositions hereof may be prepared containing amounts of hGH at least about 0.1 mg/ml, upwards of about 20 mg/ml or more, preferably from about 1 mg/ml to about 10 mg/ml. For use of these hGH compositions in administration to human patients suffering from hypopituitary dwarfism, for example, these compositions may contain from about 0.1 mg/ml to about 10 mg/ml, corresponding to the currently contemplated dosage rate for such treatment.

#### Divalent Metal Ions and Molar Ratio

The divalent metal ion suitable for use in the formulations of the present invention may be selected from zinc, cobalt or copper. The preferred metal ion for therapeutic administration is zinc. Cobalt is less preferred due to toxicity when administered in large amounts. However,

for use in stabilizing hGH in non therapeutic formulations, cobalt use is indicated. The following discussion of zinc and zinc salts may be equally applied to cobalt and copper ions. Zinc is present in the formulation as the divalent  $Zn^{+2}$  ion which may be formulated using any pharmaceutically acceptable zinc salt. For example zinc chloride, zinc acetate, zinc carbonate, zinc citrate, and the like. Most preferred is zinc chloride with a molecular weight of about 136.29 daltons (hydrated). Zinc ion is optimally present at a level sufficient to form stable zinc-hGH dimers each containing two zinc ions and two hGH molecules, when stability is the principle concern. However, when stability and hGH systemic distribution are both of concern, the zinc may be present at a molar concentration greater than 5% of the hGH molar concentration, advantageously greater than 50% of the hGH molar concentration, and most preferred is greater than 90% of the hGH molar concentration. In a specific preferred embodiment, the metal ion is zinc and the zinc:hGH molar ratio is 1:1. At this molar ratio the hGH:zinc dimer is soluble and when released into bodily fluids is rapidly distributed. The molar concentration of zinc may exceed the molar concentration of hGH, however at pH ranges above 7.0 the solubility of zinc hydroxide becomes a limiting factor. Therefore, the excess zinc ion in pH 7.0-7.4 formulations normally is less than 100  $\mu M$ , more commonly less than 50  $\mu M$ .

#### Buffer and pH

The formulation of the present preferably contains a buffer. The buffer may be any pharmaceutically acceptable buffering agent such as phosphate, tris-HCl, citrate and the like. The preferred buffer is a phosphate buffer and the molar ratio of hGH:phosphate buffer is 1:25-250, advantageously 1:50-200, most advantageously 1:75-125. A buffer concentration greater than or equal to 2.5mM and less than 20mM is preferred, most advantageously 5-10mM. In this concentration range of buffer, minimal inactivation of hGH occurs. Advantageously a sodium phosphate or tris or buffer is used. Suitable pH ranges, adjusted with buffer, for the preparation of the formulations hereof are from about 4 to about 8, advantageously about 6 to 8, most advantageously about 7.4. The formulation pH should be less than 7.5 to reduce deamidation. Above pH 7.0 the solubility of any free  $zinc^{+2}$  is reduced due to the formation of zinc hydroxide. pH values below 7.0 may result in particulate formation upon reconstitution from a lyophilized state.

#### 3 0 Formulation Amino acids

In an alternative embodiment of the present invention, a pharmaceutically acceptable amino acid, for example glycine, is added to the hGH:metal ion formulation, preferably a zinc:hGH formulation. When glycine is present, the molar ratio of hGH:glycine is 1:5-200. Glycine may inhibit the formation of inactive hGH dimers or other inactive complexes when it is added in these ratios. In addition to glycine, amino acids such as alanine, glutamine, asparagine, arginine or lysine or derivatives of such amino acids may be used in the subject formulation. Such amino acids are particularly advantageous when lyophilizing the formulation to create a sufficient mass to form a stable, dry caked formulation.

### Formulation Bulking Agents

In an alternative embodiment of the present invention, a bulking agent may be added to the formulation. The bulking agent may be any pharmaceutically acceptable agent such as a carbohydrate, for example, glucose, mannose, or dextrans; sugar alcohols such as mannitol or sorbitol. When present, the molar ratio of hGH:bulking agent may be between 1:50-8000. As an alternative to mannitol, other sugars or sugar alcohols are used such as sucrose, maltose, fructose, lactose and the like. The use of mannitol, glycine and a non-ionic surfactant in an hGH formulation and as a lyophilization bulking matrix is described in U.S. Pat. No. 5,096,885.

### Non-Ionic Surfactant

In another embodiment a non-ionic surfactant is added to the hGH-zinc formulation. The formulation of the subject invention may optionally include one of several types of non-ionic surfactants, such as the polysorbates (e.g. polysorbate 20, 80 etc.) and the poloxamers (e.g. poloxamer 188). Advantageously polysorbate 80 is used, and the molar ratio of hGH:polysorbate 80 may be 1:0.03-30. On a weight to volume basis, polysorbate 80 is added in amounts of about 0.001 to about 2% (w/v), in order to enhance further the stability of the hGH. Polysorbate 80, in concentrations above 0.01% (w/v) may reduce the amount of inactive hGH aggregates forming upon lyophilization and reconstitution. The use of non-ionic surfactants improves formulation stability when exposed to shear and surface stresses without causing denaturing of the protein. Further, such surfactant containing hGH formulations, may be employed in aerosol devices such as those used in a pulmonary dosing, and needleless jet injector guns. Such delivery formulations may be improved by the addition of non-ionic surfactants in the range of 0.1-5% (w/v).

### hGH Formulation, Storage and Administration

The increased stability of hGH formulations created by the formulations of the present invention permits a wider use of hGH formulations that may be more dilute, or alternatively, more concentrated, than those commonly in use in the absence of divalent metal ions, such as zinc. For example, the presence of zinc-hGH dimers also reduces the creation of surface induced denaturation of hGH that occurs during aerosolization or needleless injection of an hGH formulation. Further optimal dispensing of the hGH formulations may be made wherein the hGH formulations of the present invention are dispensed into vials at 1-50 mg/vial, preferably 2-25 mg/vial, and most preferably 3-10 mg/vial. The increased stability of hGH-zinc formulations permits liquid formulations and long term liquid storage, including storage below freezing and above freezing temperatures.

hGH:metal ion formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. hGH:metal ion formulations ordinarily will be stored in lyophilized form or in solution.

Therapeutic hGH:metal ion compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

5 The route of hGH:metal ion formulations is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. hGH:metal ion formulations is administered continuously by infusion or by bolus injection.

10 Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res., 15: 167-277 [1981] and Langer, Chem. Tech., 12: 98-105 [1982] or poly(vinylalcohol)], polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L- glutamate (Sidman *et al.*, Biopolymers, 22: 547-556 [1983]), non-degradable  
15 ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot<sup>TM</sup> (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

20 While polymers such as ethylene-vinyl acetate and lactic acid- glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. Sustained-release hGH:metal ion formulations compositions also include liposomally entrapped hGH:metal ion formulations. Liposomes containing hGH:metal ion formulations are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S.  
25 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal hGH:metal ion formulations therapy.

30 The increased stability of the hGH formulations of the present invention permits dispensed vials containing hGH to be stored at temperatures above freezing, particularly where refrigeration is not available, or has been interrupted. Stability at room and body temperatures facilitates administration by allowing the hGH formulations of the present invention to be warmed prior to use, and to be stored in this warmed state without substantial loss of activity prior to such administration. This permits hGH administration in clinical situations where hGH  
35 formulations must be stored, transported, and used in the absence of optimal refrigeration. Optionally, a preservative may be added where the anticipated use of the vial may compromise the sterility of the formulation. Among the preferred pharmaceutically acceptable preservatives are phenol, benzylalcohol, and para or metacresol. The improved stability of

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hGH-zinc formulations of the present invention facilitates the distribution, storage and administration of hGH not previously available for any hGH formulation.

The formulation of the present invention may be prepared by well known procedures. For example, a solution of hGH in the final formulation is prepared by buffer exchange on a gel filtration column. The elution buffer contains the zinc, buffer and any other components of the desired formulation. The concentration of the hGH protein is obtained by dilution of this resulting gel filtration eluate to a desired protein concentration. The formulation is sterile filtered, and can be stored for more than several weeks at 5°C and above., or filled into sterile vials. The vials may then be stored at 5° C., frozen, or freeze-dried using an appropriate lyophilization cycle.

In a preferred embodiment, the formulation of the subject invention comprises the following components at pH 7.4:

<u>Ingredient</u>	<u>Quantity per ml for administration (mg)</u>	<u>Molar Ratio</u>
r-hGH	1.0	1
ZnCl <sub>2</sub>	0.0062	1
NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	0.18	}
NaH <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	1.32	}110

20

or as an alternative formulation plus:

Glycine	0.34	100
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25

or as an alternative formulation plus:

Mannitol	9.0	1100
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or as an alternative formulation plus:

Polysorbate 80	0.20	3
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#### Formulation summary

Disclosed is a stabilized pharmaceutically acceptable formulation of human growth hormone comprising: a) substantially pure hGH, and b) divalent metal ion. This hGH formulation contains a divalent metal ion selected from zinc, cobalt or copper. The hGH formulation contains the divalent metal ion and hGH are in about equal molar amounts. In an one embodiment, the hGH molar concentration is in excess of the divalent metal ion molar concentration. The formulation of hGH and zinc may contain hGH wherein the concentration is greater than 0.5 micromolar and less than 2.0 millimolar. More preferably, the hGH concentration is greater than 40 micromolar and less than 1.0 millimolar. Most preferably, the

hGH concentration is greater 0.2 millimolar and less than 0.5 millimolar. The hGH:zinc dimer of the present invention may contain a zinc concentration greater than 0.5 micromolar and less than 2.0 millimolar. More preferably, the zinc concentration is greater than 40 micromolar and less than 1.0 millimolar. Most preferably, the zinc concentration is greater than 0.1 millimolar and less than 0.6 millimolar. In one preferred example, the hGH concentration is about 0.45 millimolar (10 mg/ml) and the zinc concentration is about 0.45 millimolar.

The hGH:zinc dimer formulation may contain zinc from any pharmacologically acceptable salt, for example zinc chloride, zinc acetate, zinc carbonate, or zinc citrate. The hGH:zinc formulation of the present invention may contain one or more additional formulation components selected from the following: c) a buffer, d) an amino acid, e) a bulking agent, or f) nonionic surfactant. The buffer may be any pharmacologically acceptable buffer, for example a phosphate or a tris buffer. A preferred method of stabilizing human growth hormone is combining substantially pure hGH and zinc in about equal molar amounts. A preferred method for the stable storage of a human growth hormone (hGH) and zinc formulation comprises: (a) formulating hGH and zinc in a molar ratio of about 1:1 in a pharmaceutically acceptable vehicle; (b) dispensing into a pharmaceutically acceptable storage container the formulation of step (a); and, (c) storing said container at a temperature between -76°C. and 60°C for a period of at least about 3 days. A preferred method of preventing the formation of human growth hormone inactive aggregates is accomplished by adding divalent metal ion to an hGH formulation in an amount sufficient to prevent the formation of inactive hGH aggregates. This method of preventing inactive hGH aggregates may be accomplished using divalent metal ions such as zinc, cobalt or copper.

One method of administering an aqueous human growth hormone comprises the steps of administering an aqueous formulation with an aerosol device or needleless injector gun, wherein the hGH formulation comprises (a) substantially pure hGH, and (b) zinc. This hGH formulation used in the aerosol device or needleless injector gun may contain one or more additional formulation components selected from the following: c) a buffer, d) an amino acid, e) a bulking agent, or f) nonionic surfactant. This formulation may contain a phosphate or a tris buffer, it may contain one of the following amino acids: glycine, alanine, glutamine, asparagine, arginine or lysine, it may contain one of the following bulking agents: mannitol, sorbitol, sucrose, maltose, fructose, lactose, glucose or mannose, and it may contain a nonionic surfactant is selected from the following: polysorbate 80 or poloxamer.

The methods of the present invention may be used to create stabilized formulations of helical cytokines, such as human prolactin and human placental lactogen. The formulation of a stabilized pharmaceutically acceptable formulation of human prolactin contains (a) substantially pure human prolactin, and (b) divalent metal ion. The formulation of a stabilized pharmaceutically acceptable formulation of human placental lactogen contains (a) substantially pure human placental lactogen, and (b) divalent metal ion. The formulation of prolactin or

human placental lactogen may contain a divalent metal ion is selected from zinc, cobalt or copper, preferably zinc.

#### Characterization of Zinc Stabilization of hGH

5 In the course of analyzing the binding of  $Zn^{2+}$  in the tight complex between hGH and the human prolactin binding protein (hPRLbp) ( $K_D = 0.033$  nM; B. C. Cunningham, S. Bass, G. Fuh, J. A. Wells *Science* **250**, 1709-1712 (1990)), the binding of zinc to each component was evaluated. Equilibrium dialysis showed there was no specific binding of  $^{65}Zn^{2+}$  to the hPRLbp. In contrast, Scatchard analyses of binding studies performed at two different fixed concentrations of hGH (3.3  $\mu$ M and 4.8  $\mu$ M) show that zinc forms a 1:1 complex with hGH (Fig. 1). The Scatchard  
10 plots from these and several other studies are convex indicating that the binding of one zinc atom promotes the tighter binding of another zinc atom (positive cooperativity (J. M. Boeynaems and J. E. Dumart, in *Outlines of Receptor Theory*, Elsevier/North Holland, New York, New York (1980)). From the linear portions of these plots we estimate an apparent  $K_D$  for the higher affinity zinc site to be about 1  $\mu$ M.

15 Size exclusion chromatography (Fig. 2) of hGH (at an average concentration of 15  $\mu$ M) in 1 mM EDTA or 10 mM  $MgCl_2$  shows that hGH elutes as a symmetrical peak of molecular weight ~20 kDa which corresponds to monomer hGH (molecular weight 22 kDa). However, in the presence of 50  $\mu$ M  $ZnCl_2$  (no EDTA) the hormone runs at an apparent molecular weight that is 50% larger than in EDTA. The asymmetry of this peak is characteristic of a species  
20 that is dissociating on the column (G. K. Ackers and T. E. Thompson, *Proc. Natl. Acad. Sci. U.S.A.* **53**, 342-349 1965). At higher concentrations, on equimolar mixture of hGH and  $ZnCl_2$  (at an average concentration of ~150  $\mu$ M) the complex runs as a more symmetrical peak with an apparent molecular weight that is 1.85 times the monomeric hGH peak. Thus, at higher concentrations the dimerization is essentially complete. At much lower concentrations (average  
25 0.15  $\mu$ M hGH, 50  $\mu$ M  $ZnCl_2$ ) hGH runs only as a monomer. These data indicate that at micromolar concentrations of hGH, zinc is able to promote the formation of an hGH dimer.

Sedimentation equilibrium studies (Fig. 3) confirm that hGH forms a dimeric complex (~44 kDa) in the presence of but not the absence of 20  $\mu$ M  $ZnCl_2$ . Moreover, the concentration dependence for the dimer formation allows us to calculate an apparent dimerization equilibrium  
30 constant for hGH of 2.6  $\mu$ M in 20  $\mu$ M  $ZnCl_2$  (Table I). To begin to evaluate the structure of the zinc sites in the hGH dimer we used  $Co^{2+}$ , a spectroscopically active metal (I. Bertini and C. Luchinat, *Adv. Inorg. Biochem.* **6**, 71-111 (1984); M. T. Martin, B. Holmquist, J. F. Riordan, *J. Inorg. Chem.* **36**, 27-37 (1989)), in place of  $Zn^{2+}$ . Gel filtration experiments similar to those  
35 above show that  $Co^{2+}$  induces dimerization of hGH. Moreover, both  $Zn^{2+}$  and  $Co^{2+}$  induce very similar UV difference spectra upon dimerization of the hormone (Fig. 4A). We believe the slight differences between the two metals in the far UV region of the spectra reflects spectroscopy of the metal complexes and not differences in their coordination.

Titration of hGH with  $Co^{2+}$  produces a  $Co^{2+}$  visible absorption spectrum with a maximum at 525 nm and molar absorptivity of 260  $cm^{-1} M^{-1}$  (Fig. 4B). The wavelength



maximum and molar absorptivity is characteristic of a  $\text{Co}^{2+}$  in a tetrahedral environment coordinated by a mixture of nitrogen and oxygen containing ligands (I. Bertini and C. Luchinat, *Adv. Inorg. Biochem.* **6**, 71-111 (1984); M. T. Martin, B. Holmquist, J. F. Riordan, *J. Inorg. Chem.* **36**, 27-37 (1989)). The circular dichroic spectrum of the  $\text{Co}^{2+}$ -hGH dimeric complex is virtually

5 flat indicating that the sum of all bound cobalt is in a symmetrical environment (M. T. Martin, B. Holmquist, J. F. Riordan, *J. Inorg. Chem.* **36**, 27-37 (1989)). This suggests either that each cobalt site in the dimer is highly symmetrical, or more likely that the two cobalt sites are asymmetric but pseudo-mirror images of each other so that the dichroic effect of one is equal and of opposite sign thus canceling each other.

10         Mutational analysis was used to further localize the sites of zinc coordination in the  $(\text{Zn}^{2+}\text{-hGH})_2$  complex. Previous studies indicated that His18, His21, and Glu174 are involved in zinc mediated binding of hGH to the hPRLbp (B. C. Cunningham, S. Bass, G. Fuh, J. A. Wells *Science* **250**, 1709-1712 (1990)). Indeed, mutation of each of these residues to alanine causes a substantial reduction in formation of dimeric hGH by gel filtration (Fig. 5). From the elution of  
15 the protein in the presence of  $\text{ZnCl}_2$  or EDTA, the (E174A) variant gives no indication of dimer formation, and H21A and H18A exhibit substantially reduced dimer formation.

Sedimentation equilibrium studies allowed us to quantify the effect of mutating residues in an around the putative zinc site on the dimerization constant of hGH in  $20\ \mu\text{M}\ \text{ZnCl}_2$  (Table I). The dimerization affinity is about 150-fold weaker in the presence of EDTA compared to  $\text{ZnCl}_2$ .  
20 Mutations of His21 or Glu174 to alanine produce reductions in the dimer affinities of 58-fold, and 11-fold, respectively. There is essentially no effect for mutating Asp171 which is nearby the zinc site.

Equilibrium dialysis studies show that the reduction in dimer formation correlates with a loss in capacity to bind  $^{65}\text{Zn}^{2+}$  (Table 2). At low concentrations of  $\text{Zn}^{2+}$ , the ratio of bound to  
25 free zinc decreases in the following order: hGH ~ D171A >> H21A ~ H18A > E174A. Thus, gel filtration, sedimentation equilibria, and equilibrium dialysis experiments indicate that His18, His21 and Glu174 are involved in coordinating zinc and promoting formation of the hormone dimer. These residues are near to each other in the tertiary structure of hGH (Fig 6A).

Zinc typically coordinates four ligands in proteins (B. L. Vallee and D. S. Auld., *Proc. Natl. Acad. Sci. U. S. A.* **87**, 220-224 (1990)). Sedimentation equilibrium and zinc binding studies  
30 data indicate that Asp171 is not the fourth zinc ligand even though it is close by in the folded model of hGH. There are no other nearby side chains that could coordinate zinc. However, it is possible that the fourth zinc ligand is a water molecule. Alternatively, it is possible that His18, His21, or Glu174 from each hGH molecule bridge the two  $\text{Zn}^{2+}$  atoms in the dimer (for example  
35 see Fig. 6B). In this model, each monomer of hGH in the dimer is related by a two-fold symmetry axis with the zinc atoms sandwiched at the interface. This would explain the absence of a strong  $\text{Co}^{2+}$  circular dichroic effect in the dimer; a pseudo-mirror plane of symmetry created by the "twinned" metal sites would create equal and opposite dichroic effects. Furthermore, the highly cooperative nature of  $\text{Zn}^{2+}$  binding suggests that the two sites

are interdependent. Such a ligand bridged bi-metal site is not a natural precedent. For example, Cu-Zn superoxide dismutase contains a His residue that coordinates to both the copper and zinc (J. A. Tainer, E. D. Getzoff, J. S. Richardson, D. C. Richardson, *Nature* 306, 284-287 (1983)).

Growth hormone can be isolated in a multitude of depending of the conditions and source of the pituitary (for review see U. J. Lewis, *Annu. Rev. Physiol.* 46, 33-42 (1984).). Non-covalently associated dimers containing 9 different molecular weight hGH related polypeptides (8-22kD) were isolated. Although the nature of these charge isomer dimers was not well characterized, they were isolated and studied in the absence of zinc following dialysis and chromatographic elution from an ion-exchange resin which removed any zinc that may have been present (P. Brostedt and P. Roos, *Prep. Biochem.* 19, 217-229 (1989)). We propose that the  $\text{Zn}^{2+}$ -hGH dimer produced here *in vitro* is the major storage form of hGH *in vivo*. A stable formulation of hGH and zinc therefore results in a therapeutic complex analogous to what the pituitary releases. Normal human pituitary glands (~1 ml volume) contain 2 to 4 mgs of hGH (W. F. Daughaday, in *Textbook of Endocrinology*, seventh edition, Chapter 18, ed. J. D. Wilson and D. W. Foster, (W. B. Saunders Co., Philadelphia, 1985), p. 577). The zinc content in rat pituitary has been estimated to be 75 to 100 ng/mg dry weight (O. Thorlacius-Ussing, *Neuroendocrinol.* 45, 233-242 (1987).) which corresponds to about 10  $\mu\text{g}$  total Zn/ml. Thus, in a pituitary gland there is 160 nmol of zinc and 90 to 180 nmol of hGH. Most of the zinc in the pituitary is located in somatotrophic granules (O. Thorlacius-Ussing, (1987).) along with hGH (W.F. Daughaday, 1985). Furthermore, the amount of free zinc in granules (estimated by that which can be chelated with dithiozone) is about 3.4% of the total or ~50  $\mu\text{M}$ . This concentration is roughly 50-fold above that necessary to saturate the  $\text{Zn}^{2+}$  site in hGH (~1  $\mu\text{M}$ ). In addition, the total concentration of hGH in vesicles is about 2 to 4 mM. This concentration is about 1000-fold greater than the dissociation constant for the  $\text{Zn}^{2+}$ -hGH dimer (2.6  $\mu\text{M}$ ). Thus zinc and hGH are localized in somatotrophic vesicles in roughly equimolar amounts and with high enough affinity that virtually all of the hGH would exist as a  $(\text{Zn}^{2+}\text{-hGH})_2$  complex.

We suggest that the  $(\text{Zn}^{2+}\text{-hGH})_2$  complex serves at least two important functions. Firstly, the  $\text{Zn}^{2+}$ -hGH dimer is significantly more stable to denaturation than monomeric hGH as shown by its much greater resistance to unfolding in Gu-HCl (Fig. 7). In the presence of 25  $\mu\text{M}$   $\text{ZnCl}_2$ , the concentration of Gu-HCl at which 50% of the hGH is denatured increases from 4.1 M to 4.8 M. hGH is released in pulses from the pituitary every 4 to 12 h depending upon the age, sex and health of the individual (A. L. Taylor, J. L. Finster, D. H. Mintz, *J. Clin. Invest.* 48, 2349 (1969); R. G. Thompson, A. Rodriguez, A. Kowarski, and R. M. Blizzard *J. Clin. Invest.* 51, 3193 (1972); K. Y. Ho, W. S. Evans, R. M. Blizzard, J. D. Veldhuis, G. R. Merriam, E. Samojlik, R. Furlanetto, A. D. Rogol., D. L. Kaiser, M. O. Thomer, *J. Clin. Endocrinol. Metab.* 64, 51-58 (1987)). This means that the hormone can be stored for perhaps days at 37°C in vesicles before it is finally released. Our studies suggest that the  $(\text{Zn}^{2+}\text{-hGH})_2$  complex in or out of vesicles is substantially more resistant to denaturation, aggregation and/or proteolysis than hGH in the absence of zinc.

Secondly, the concentration of hGH when released from the pituitary is above 1 mM, yet by the time it reaches receptors in the periphery its peak concentration is about 5 nM (A. L. Taylor, J. L. Finster, D. H. Mintz, *J. Clin. Invest.* **48**, 2349 (1969); R. G. Thompson, A. Rodriguez, A. Kowarski, and R. M. Blizzard *J. Clin. Invest.* **51**, 3193 (1972); K. Y. Ho, W. S. Evans, R. M. Blizzard, J. D. Veldhuis, G. R. Merriam, E. Samojlik, R. Furlanetto, A. D. Rogol, D. L. Kaiser, M. O. Thoner, *J. Clin. Endocrinol. Metab.* **64**, 51-58 (1987)), nearly a  $10^6$ -fold dilution. Almost all cells contain hGH receptors and/or hPRL receptors. This enormous concentration gradient could result in saturating cellular receptors proximal to the pituitary. However, the receptor binding epitopes on hGH for both the hGH receptor (B. C. Cunningham and J. A. Wells, *Science* **244**, 1081-1085 (1989)) and hPRL receptor are covered in the  $(\text{Zn}^{2+}\cdot\text{hGH})_2$  complex (Fig. 6A). Therefore, we do not expect the hormone to become bioavailable until the hGH concentration approaches its dimerization dissociation constant ( $\sim 3 \mu\text{M}$ ). We suggest that the  $(\text{Zn}^{2+}\cdot\text{hGH})_2$  dimer has the added advantage of dampening stimulatory effects arising from locally high concentrations of hGH near the pituitary gland.

Zinc is known to be important for the storage of insulin (J. C. Hutton, *Experientia* **40**, 1091-1104 (1984); G. Gold, and G. M. Grodsky, *Experientia* **40**, 1091-1104 (1984)). Our data provide a molecular mechanism for the importance of zinc to the storage and release of hGH that may be much more general. For example, human placental lactogen (hPL) which is 85% identical to hGH has been shown to bind to the hPRL receptor in a zinc dependent fashion and contains His18, His21, and Glu174. hPRL (and prolactins from almost all other species) contain His18, His21 and Asp174 (for review see C. S. Nicoll, G. L. Mayer, S. M. Russell, *Endocrine Reviews* **7**, 169 (1986)). Addition of small amounts of zinc will dimerize hPL or precipitate hPRL at concentrations of hormone and metal above about  $10 \mu\text{M}$ . Furthermore, non-primate growth hormones contain Gln18 instead of His18, His21, and Glu174. Glutamine is not a known ligand for zinc in proteins, but amides can coordinate metals. In any case, we find that the H18A mutation still retains a dimerization dissociation constant of  $150 \mu\text{M}$ , which is still below the likely concentration of growth hormone in somatotrophic granules ( $>1 \text{ mM}$ ). Alternatively, all non-primate growth hormones contain His171 and it is conceivable that this serves the role of the missing His18. Although we do not believe Asp171 coordinates zinc in the  $(\text{Zn}^{2+}\cdot\text{hGH})_2$  complex, His is chemically a stronger ligand for zinc. We have not tested D171H as an alternative in wild-type hGH or the H18A variant. Finally, hGH is structurally similar to the family of helical cytokines including interleukin-2, colony stimulating factor, interferon- $\beta$  and others (for review see D. A. D. Parry, E. Minasian, S. J. Leach, *J. Mol. Recog.* **1**, 107-110 (1988)). It is believed that some of these proteins may be stored as complexes with zinc prior to their release from cells.

#### Use of Zinc Binding Site in Other Polypeptide hormones.

We suggest that zinc (cobalt or copper) induced hormone oligomerization can modulate in a reversible fashion the stability and activity of the somato-lactogenic family of hormones and perhaps other helical cytokines. Specifically the formulations of the present invention may be

prepared with human prolactin or human placental lactogen in place of the hGH. Both of these human helical cytokines contain a divalent metal binding site, preferentially zinc. Those cytokines which do not contain such a zinc complexing site can be modified to contain an hGH-like zinc binding site using site specific mutagenesis procedures well known in the art, thereby  
5 allowing the formation of stable zinc:helical cytokine complexes suitable for stable formulation. Such a zinc binding site may be constructed by placing two histidines and one Glutamate between two polypeptide helicies adjacent in the same structural arrangement as in hGH.

A zinc binding site, for example a site analogous to hGH amino acids His18, His21 and Asp174, can be introduced into the three dimensional structure of other helical cytokines.  
10 Among such helical cytokines are the interleukins, the interferons, polypeptide hormones, such as growth hormones, prolactin, and placental lactogen. The actual location of such zinc binding amino acids need not be in the same linear position as in hGH. Rather, amino acids on the helical cytokine surface would need to specify analogous functional positions for binding the divalent metal ion between two helical regions of the cytokine polypeptides. For example, the helical  
15 cytokine amino acid sequence may be modified to create a zinc binding site so that two of the helical cytokines complex with two zinc (or other divalent metal ions), to form a stable dimer.

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary, is intended to cover various modifications and equivalent formulations included within the spirit and scope of  
20 the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalent formulations. The following examples are intended to illustrate the best mode now known for practicing the invention, but the invention is not to be considered limited to these examples.

#### EXAMPLE 1

##### 25 CHARACTERIZATION OF hGH DIMERS

The properties of normal hGH dimers formed by zinc chloride and hGH were evaluated by equilibrium dialysis, gel filtration, sedimentation equilibrium analysis, UV difference spectra, and visible light difference spectra. The ability of variant hGH molecules to form dimers in the presence of zinc were evaluated by gel filtration analyses.

30 Figure 1 shows the equilibrium dialysis and Scatchard analysis for binding of  $^{65}\text{Zn}^{2+}$  to hGH ( $\circ$  3.3  $\mu\text{M}$  final or  $\bullet$  4.8  $\mu\text{M}$  final). Precautions were taken to minimize zinc contamination in the buffers and dialysis cells as previously described (B. C. Cunningham, S. Bass, G. Fuh, J. A. Wells *Science* 250, 1709-1712 (1990)). hGH was mixed with dialysis buffer containing 20 mM Tris (pH 7.5), 140 mM NaCl and 10 mM  $\text{MgCl}_2$ . The  $\text{MgCl}_2$  was added to  
35 reduce non-specific binding of  $\text{Zn}^{2+}$  to the dialysis cell membrane and hGH. The  $\text{MgCl}_2$  neither promoted nor inhibited dimerization of hGH. Aliquots (100  $\mu\text{l}$ ) of the solution containing hGH (3.3 or 4.8  $\mu\text{M}$ ) was added to one side of the dialysis cell. Serial dilutions (in triplicate) of cold  $\text{ZnCl}_2$  containing 0.3  $\mu\text{M}$   $^{65}\text{ZnCl}_2$  (8 dilution steps ranging from 0 to 40  $\mu\text{M}$  final) were added to the other side of the dialysis cell. Cells were rotated slowly for 16-20 h at 25°C. Control

experiments determined this was sufficient time to reach equilibrium. Aliquots (50  $\mu$ l) from each side of the dialysis cell were diluted into scintillation cocktail and analyzed for  $^{65}\text{Zn}^{2+}$  content. From these values the concentrations of bound and free zinc were calculated. Error bars indicate standard deviations from triplicate determinations.

5        The Scatchard plots from these and several other studies bend upward indicating that the binding of one zinc atom promotes the tighter binding of another zinc atom (positive cooperativity). From the linear portions of these plots we estimate an apparent  $K_D$  for the higher affinity zinc site to be about 1  $\mu$ M

10        Figure 2 shows the gel filtration chromatography of hGH in the presence (A) or absence (B) of  $\text{ZnCl}_2$ . One hundred microliters of a solution containing 50  $\mu$ M hGH, 20 mM Tris (pH 7.5) 100 mM NaCl, and either 50  $\mu$ M  $\text{ZnCl}_2$  or 1 mM EDTA was applied to a Superose 12 column and eluted at 0.35 ml/min with the same buffer without hGH. Size standards (bovine  $\gamma$ -globulin, ovalbumin and myoglobin) were run separately to calibrate the column, and their peak locations are indicated in the standard curve at the top of the chromatogram. These data indicate that  
15        at micromolar concentrations of hGH, zinc is able to promote the formation of an hGH dimer.

20        Figure 3 shows the sedimentation equilibrium analysis of hGH in the presence (●) or absence (○) of 20  $\mu$ M  $\text{ZnCl}_2$ . Samples of hGH (9.1  $\mu$ M, 6.8  $\mu$ M or 4.5  $\mu$ M) in 10 mM Tris (pH 8.0), 0.1 M NaCl with or without 20  $\mu$ M  $\text{ZnCl}_2$  were loaded into a 6-channel Yphantis cell (26), and centrifuged in a Beckman Model E centrifuge at 20,000 rpm for 48 h at 20°C. Panel A shows the concentration of hGH (mg/ml) versus radial distance. The data combined for all three hormone concentrations) was fit to a monomer-dimer equilibrium to determine the dimerization constant ( $K_D = 2.6 \mu$ M). Panel B shows  $\ln [\text{hGH concentration}]$  vs  $r^2$  which gives a linear plot in the absence of  $\text{ZnCl}_2$  (○), as expected for a monomeric species. The plot is curvilinear in the presence of  $\text{ZnCl}_2$  (●) showing self association. These sedimentation  
25        equilibrium studies confirm that hGH forms a dimeric complex (~44 kDa) in the presence of, but not the absence of 20  $\mu$ M  $\text{ZnCl}_2$ . Moreover, the concentration dependence for the dimer formation allows us to calculate an apparent dimerization equilibrium constant for hGH of 2.6  $\mu$ M in 20  $\mu$ M  $\text{ZnCl}_2$  (Table I).

30        In Table 1, the dimerization constants of hGH and hGH mutants are shown that resulted from by analytical sedimentation equilibrium analysis. The analysis of each of these hormones was carried out as described for Fig. 3.

Table 1 Dimerization Constants

	Hormone	$K_D$ ( $\mu$ M)
5	hGH + 20 $\mu$ M $ZnCl_2$	26
	hGH + 1 mM EDTA	380
	H21A + 20 $\mu$ M $ZnCl_2$	150
	E174A + 20 $\mu$ M $ZnCl_2$	28
	D171A + 20 $\mu$ M $ZnCl_2$	3.9

10

To evaluate the structure of the zinc sites in the hGH dimer we used  $Co^{2+}$ , a spectroscopically active metal, in place of  $Zn^{2+}$ . Gel filtration experiments similar to those above show that  $Co^{2+}$  induces dimerization of hGH. Moreover, both  $Zn^{2+}$  and  $Co^{2+}$  induce very similar UV difference spectra upon dimerization of the hormone as shown in Fig. 4A. In Figure 4A are shown the UV difference spectra for binding of  $Zn^{2+}$  (—) and  $Co^{2+}$  (---) to hGH. Tandem cells contained on one side hGH (45  $\mu$ M final) and buffer (25 mM Tris (pH 7.5), 140 mM NaCl) plus either 50  $\mu$ M  $ZnCl_2$  or 50  $\mu$ M  $CoCl_2$ . The other side contained just buffer plus either 50  $\mu$ M  $ZnCl_2$  or 50  $\mu$ M  $CoCl_2$ .

Titration of hGH with  $Co^{2+}$  produces a  $Co^{2+}$  visible absorption spectrum with a maximum at 525 nm and molar absorptivity of 260  $cm^{-1} M^{-1}$  (Fig. 4B). The wavelength maximum and molar absorptivity is characteristic of a  $Co^{2+}$  in a tetrahedral environment coordinated by a mixture of nitrogen and oxygen containing ligands. The circular dichroic spectrum of the  $Co^{2+}$ -hGH dimeric complex is virtually flat indicating that the sum of all bound cobalt is in a symmetrical environment. This suggests either that each cobalt site in the dimer is highly symmetrical, or more likely that the two cobalt sites are asymmetric but pseudo-mirror images of each other so that the dichroic effect of one is equal and of opposite sign thus canceling each other. Shown in Figure 4B is the visible difference spectra for binding of increasing concentrations of  $Co^{2+}$  to hGH. The experiment was identical to that in panel A except that the [hGH] was 23  $\mu$ M and [ $Co^{2+}$ ] ranged from 5 to 1250  $\mu$ M. The molar absorptivity (260  $cm^{-1} M^{-1}$ ) was calculated from the maximal change in absorbance at 525 nm ( $6 \times 10^{-3} cm^{-1}$ ) divided by the concentration of hGH in the cuvette (23  $\mu$ M).

## EXAMPLE 2

## METAL BINDING SITE OF hGH

The zinc binding site on hGH was evaluated through the use of mutant hGH molecules and equilibrium dialysis. Figure 5 shows dimerization of hGH (wild type) and hGH mutants by addition of zinc. Gel filtration analysis was performed as described for Figure 2 except that hGH and  $ZnCl_2$  were applied at a concentration of 10  $\mu$ M. Panels A-D show elution profiles for hGH and the designated hGH mutants. Mutants are indicated by the single-letter amino acid for the wild-type residue followed by its position in mature hGH and then the mutant residue. For

example, H18A designates a mutant where His18 is changed to Ala. Mutants of hGH were expressed in *Escherichia coli* and purified as previously described. Within each panel are superimposed gel filtration chromatograms run in buffers containing a fixed concentration of either 10  $\mu\text{M}$   $\text{ZnCl}_2$  (shaded peaks) or 1 mM EDTA (unshaded peaks).

5 This mutational analysis was used to further localize the sites of zinc coordination in the  $(\text{Zn}^{2+}\text{-hGH})_2$  complex. Previous studies indicated that His18, His21, and Glu174 are involved in zinc mediated binding of hGH to the hPRLbp (B. C. Cunningham, S. Bass, G. Fuh, J. A. Wells *Science* 250, 1709-1712 (1990)). From the elution of the protein in the presence of  $\text{ZnCl}_2$  or EDTA, the (E174A) variant gives no indication of dimer formation, and H21A and H18A exhibit  
10 substantially reduced dimer formation. These sedimentation equilibrium studies allowed us to quantify the effect of mutating residues in an around the putative zinc site on the dimerization constant of hGH in 20  $\mu\text{M}$   $\text{ZnCl}_2$  (Table I above). The dimerization affinity is about 150-fold weaker in the presence of EDTA compared to  $\text{ZnCl}_2$ . Mutations of His21 or Glu174 to alanine produce reductions in the dimer affinities of 58-fold, and 11-fold, respectively. There is  
15 essentially no effect for mutating Asp171 which is nearby the zinc site.

Equilibrium dialysis studies show that the reduction in dimer formation correlates with a loss in capacity to bind  $^{65}\text{Zn}^{2+}$  (Table 2). In Table 2 the comparative binding of low concentrations of  $\text{ZnCl}_2$  (0.6  $\mu\text{M}$  final) to hGH and hGH mutants are shown. Equilibrium dialysis for binding of  $^{65}\text{Zn}_2$  to hGH and variants of hGH was carried out as previously described for Fig.  
20 1 except that the hormone concentration was increased to 45  $\mu\text{M}$  to enhance the signal and accuracy for measuring weak binding of  $\text{Zn}^{2+}$ . The H18A, H21A, D171A and E174A mutants were prepared and purified as previously described

Table 2 Comparative Binding

25	Average ( $\pm$ S.D.)	
	Hormone	$\text{Zn}^{2+}$ bound/free
	Wild-type	$18.3 \pm 4.3$
	H18A	$5.1 \pm 0.2$
30	H21A	$8.7 \pm 1.2$
	D171A	$27.4 \pm 0.2$
	E174A	$1.8 \pm 0.1$

Zinc typically coordinates four ligands in proteins (B. L. Vallee and D. S. Auld, D. S. *Proc. Natl. Acad. Sci. U. S. A.* 87, 220-224 (1990)). Sedimentation equilibrium and zinc binding studies data indicate that Asp171 is not the fourth zinc ligand even though it is close by in the folded model of hGH. There are no other nearby side chains that could coordinate zinc. However, it is possible that the fourth zinc ligand is a water molecule. Alternatively, it is possible that His18, His21, or Glu174 from each hGH molecule bridge the two  $\text{Zn}^{2+}$  atoms in the  
35

20

dimer (for example see Fig. 6B). In this model, each monomer of hGH in the dimer is related by a two-fold symmetry axis with the zinc atoms sandwiched at the interface. Shown in Figure 6, in Panel A, is the folding model for hGH based upon a 2.8 Å structure of porcine GH showing the putative location of the  $Zn^{2+}$  ligands (starred) and the extent of the prolactin receptor (dashed ellipse) and hGH receptor (solid circle).

Panel B shows one of three possible bridged ligand models that can account for the involvement of His18, His21, and Glu174 in coordinating 2 zinc atoms per hGH dimer. A salient feature of this model is the two-fold symmetry axis through the center of the "twinned" metal site. Glu174 from each hormone is shown coordinating both  $Zn^{2+}$  atoms simultaneously.

Alternatively, it is also possible that either His18 or His21 could bridge both zinc atoms instead of Glu174.

### EXAMPLE 3

#### IMPROVED hGH STABILITY

We believe that the  $(Zn^{2+}\cdot hGH)_2$  complex serves the important function of stabilizing hGH. The  $Zn^{2+}\cdot hGH$  dimer is significantly more stable to denaturation than monomeric hGH as shown by its much greater resistance to unfolding in Gu-HCl (Fig. 7). In the presence of 25  $\mu M$   $ZnCl_2$ , the concentration of Gu-HCl at which 50% of the hGH is denatured increases from 4.1 M to 4.8 M. Our studies indicate that a therapeutic formulation containing the  $(Zn^{2+}\cdot hGH)_2$  complex will be substantially more resistant to denaturation, aggregation and/or proteolysis.

Figure 7 shows the stability of hGH to denaturation by Guanidine-HCl in the presence (●) or absence (○), of 25  $\mu M$   $ZnCl_2$ . A solution containing 30  $\mu g$  hGH, 0.01 M Tris buffer pH 8.0, with or without 25  $\mu M$   $ZnCl_2$  and the indicated GuHCl concentration was equilibrated for 2 min at 25°C prior to measuring the  $\theta_{222}$  in a 1 cm CD cell. For those samples containing  $Zn^{2+}$ , the  $Zn^{2+}$  was added before the GuHCl. The fraction folded was calculated from the fractional change in  $\theta_{222}$  in the absence of Gu-HCl (folded) relative to that in 6.5 M Gu-HCl (unfolded).

### EXAMPLE 4

#### hGH FORMULATION

A stable formulation of hGH is prepared by first combining a zinc chloride solution with an hGH sample, dialyzing the hGH:zinc dimer sample against formulation buffer (5mM phosphate, pH 7.4, 50  $\mu M$  zinc chloride), diluting and dispensing into vials. A solution of zinc chloride (1mM) is slowly added directly with stirring to the hGH sample (20mg/ml) to achieve a 1:1 molar ratio, while maintaining the pH at 7.0-7.4 using phosphate buffer (1.0 M, pH 7.4). Next, the hGH:zinc dimer is dialyzed (8 hr, 4° C, hGH:buffer (v/v) 1:1000) against an aqueous formulation buffer containing zinc chloride (50  $\mu M$ ) and a sodium phosphate buffer (5 mM) equilibrated at pH 7.4. The hGH:zinc dimer mixture is then diluted by formulation buffer to a concentration of 5 mg/ml hGH. The solution is sterile filtered, and stored at 4°C until needed. then 1.0 ml is dispensed into sterile vials.



## WHAT IS CLAIMED IS:

1. A stabilized pharmaceutically acceptable formulation of human growth hormone (hGH) comprising:
  - 5 a) substantially pure hGH, and
  - b) divalent metal ion.
2. The formulation of claim 1 wherein said divalent metal ion is selected from zinc, cobalt or copper.
3. The formulation of claim 1 wherein said divalent metal ion and hGH are in about equal
- 10 molar amounts.
4. The formulation of claim 1 wherein the hGH molar concentration is in excess of said divalent metal ion molar concentration.
5. The formulation of claim 1 wherein the hGH concentration is greater than 0.5 micromolar and less than 2.0 millimolar.
- 15 6. The formulation of claim 5 wherein the hGH concentration is greater than 40 micromolar and less than 1.0 millimolar.
7. The formulation of claim 6 wherein the hGH concentration is greater 0.2 millimolar and less than 0.5 millimolar.
8. The formulation of claim 2 wherein the zinc concentration is greater than 0.5 micromolar
- 20 and less than 2.0 millimolar.
9. The formulation of claim 8 wherein the zinc concentration is greater than 40 micromolar and less than 1.0 millimolar.
10. The formulation of claim 9 wherein the zinc concentration is greater than 0.1 millimolar and less than 0.6 millimolar.
- 25 11. The formulation of claim 10 wherein the hGH concentration is about 0.45 millimolar (10 mg/ml) and the zinc concentration is about 0.45 millimolar.
12. The formulation of claim 2 wherein said zinc is selected from the following: zinc chloride, zinc acetate, zinc carbonate, or zinc citrate.
13. The formulation of claim 1 wherein one or more additional formulation components are
- 30 present and selected from the following:
  - c) a buffer,
  - d) an amino acid,
  - e) a bulking agent, or
  - f) nonionic surfactant.
- 35 14. The formulation of claim 13 wherein said buffer is selected from the following: a phosphate or a tris buffer.
15. The formulation of claim 14 wherein said buffer is a phosphate buffer.
16. The formulation of claim 13 having a pH of 4-8.

17. The formulation of claim 13 wherein said amino acid is selected from the following: glycine, alanine, glutamine, asparagine, arginine or lysine.
18. The formulation of claim 13 wherein said bulking agent is selected from the following: mannitol, sorbitol, sucrose, maltose, fructose, lactose, glucose or mannose.
- 5 19. The formulation of claim 13 wherein said nonionic surfactant is selected from the following: polysorbate 80 or poloxamer,
20. A method of stabilizing human growth hormone (hGH) comprising combining substantially pure hGH and a divalent metal ion in about equal molar amounts.
21. A method for the stable storage of a human growth hormone (hGH) and zinc
- 10 formulation comprising:
- (a) formulating hGH and zinc ion in a molar ratio of about 1:1 in a pharmaceutically acceptable vehicle;
  - (b) dispensing into a pharmaceutically acceptable storage container the formulation of step (a); and,
  - 15 (c) storing said container at a temperature between -76°C. and 60°C for a period of at least about 1 week.
22. A method of preventing the formation of human growth hormone (hGH) inactive aggregates comprising adding divalent metal ion to an hGH formulation in an amount sufficient to prevent the formation of inactive hGH aggregates.
- 20 23. The method of claim 22 wherein said divalent metal ion is selected from the following: zinc, cobalt or copper.
24. A method of administering an aqueous human growth hormone comprising the steps of: administering said aqueous formulation with an aerosol device or needleless injector gun, wherein the formulation comprises:
- 25 (a) substantially pure hGH, and
- (b) zinc ion.
25. The method of claim 24 wherein one or more additional formulation components are present and selected from the following:
- c) a buffer,
  - 30 d) an amino acid,
  - e) a bulking agent, or
  - f) nonionic surfactant.
26. The formulation of claim 25 wherein said buffer is selected from the following: a phosphate or a tris buffer.
- 35 27. The formulation of claim 25 wherein said amino acid is selected from the following: glycine, alanine, glutamine, asparagine, arginine or lysine.
28. The formulation of claim 25 wherein said bulking agent is selected from the following: mannitol, sorbitol, sucrose, maltose, fructose, lactose, glucose or mannose.

29. The formulation of claim 25 wherein said nonionic surfactant is selected from the following: polysorbate 80 or poloxamer.
30. A stabilized pharmaceutically acceptable formulation of human prolactin comprising:
- 5       a) substantially pure human prolactin, and  
      b) divalent metal ion.
31. The formulation of claim 30 wherein said divalent metal ion is selected from zinc, cobalt or copper.
32. A stabilized pharmaceutically acceptable formulation of human placental lactogen comprising:
- 10       a) substantially pure human placental lactogen, and  
      b) divalent metal ion.
33. The formulation of claim 32 wherein said divalent metal ion is selected from zinc, cobalt or copper.

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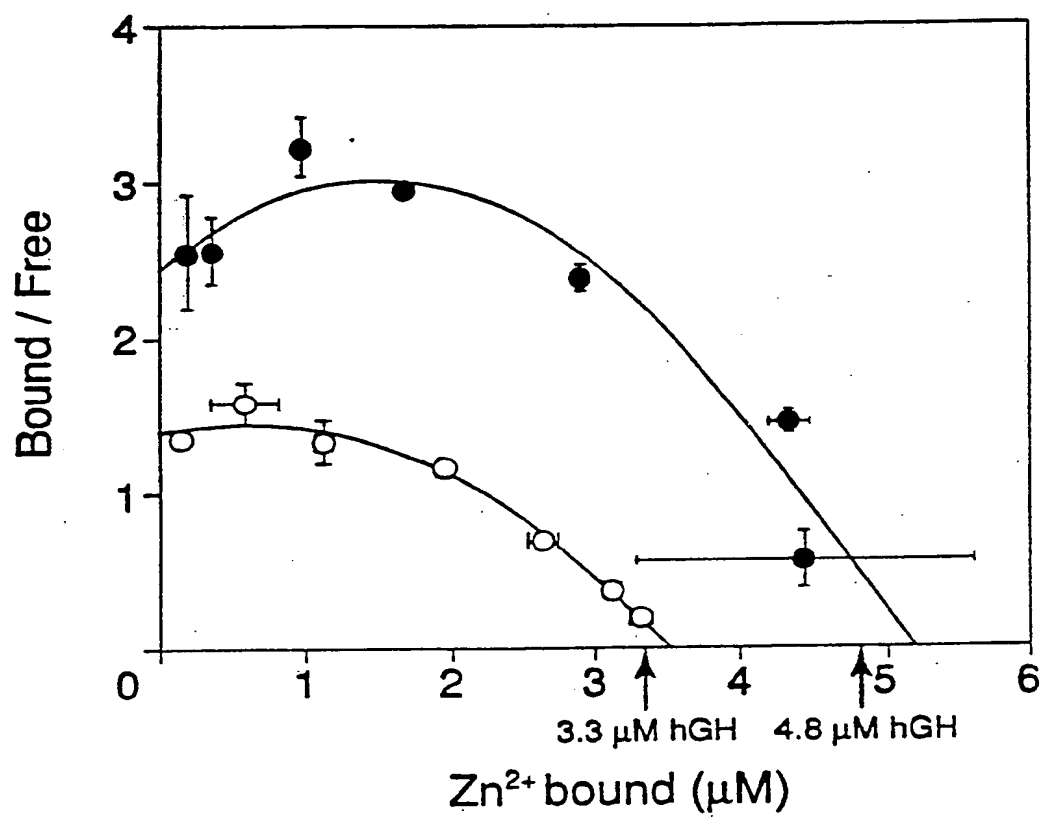


FIG. 1

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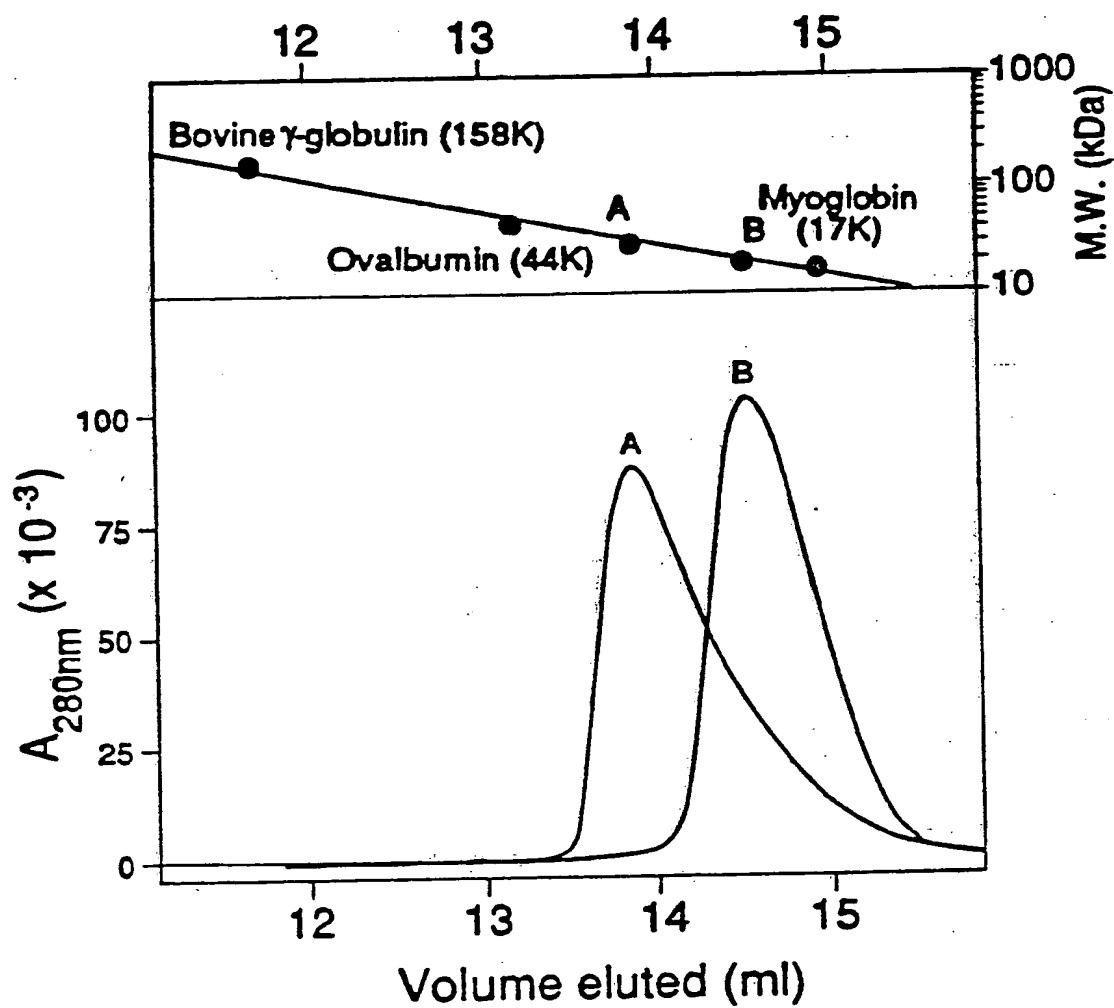


FIG. 2

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FIG. 3A

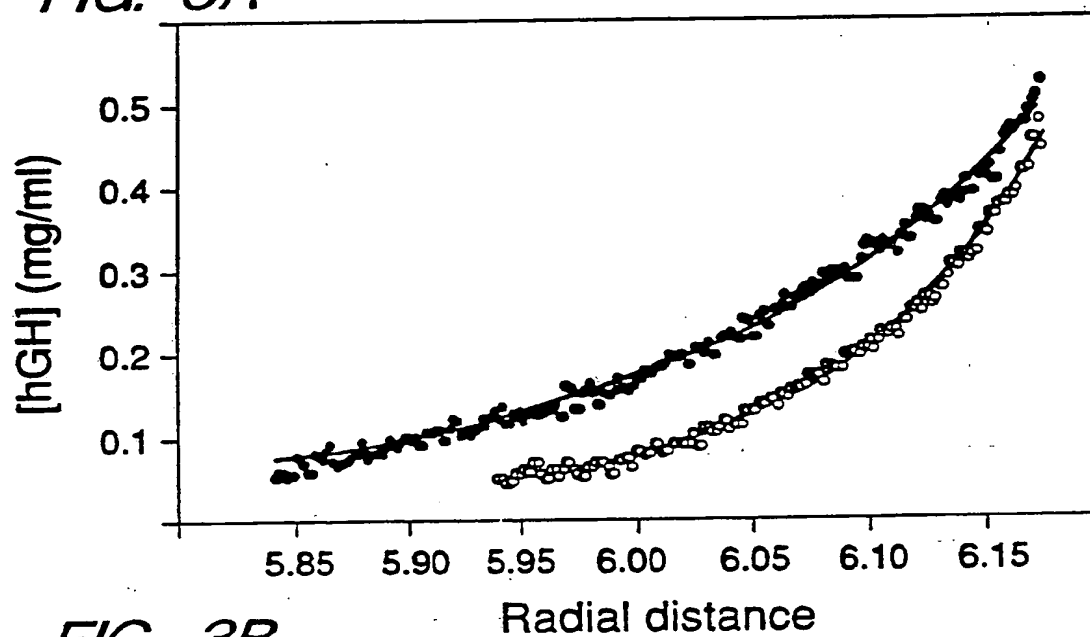
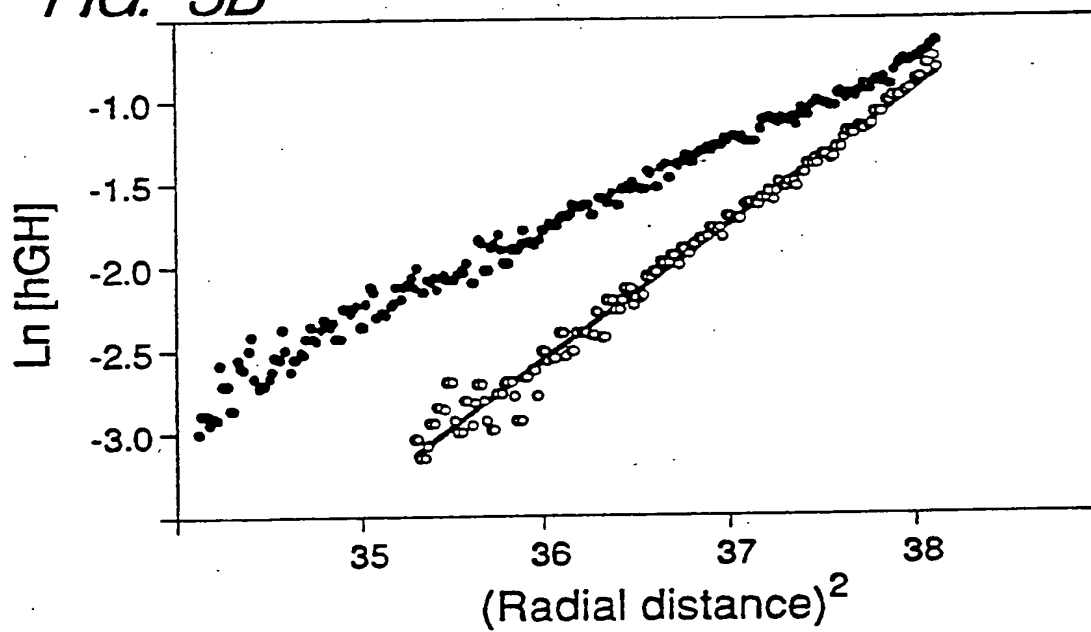
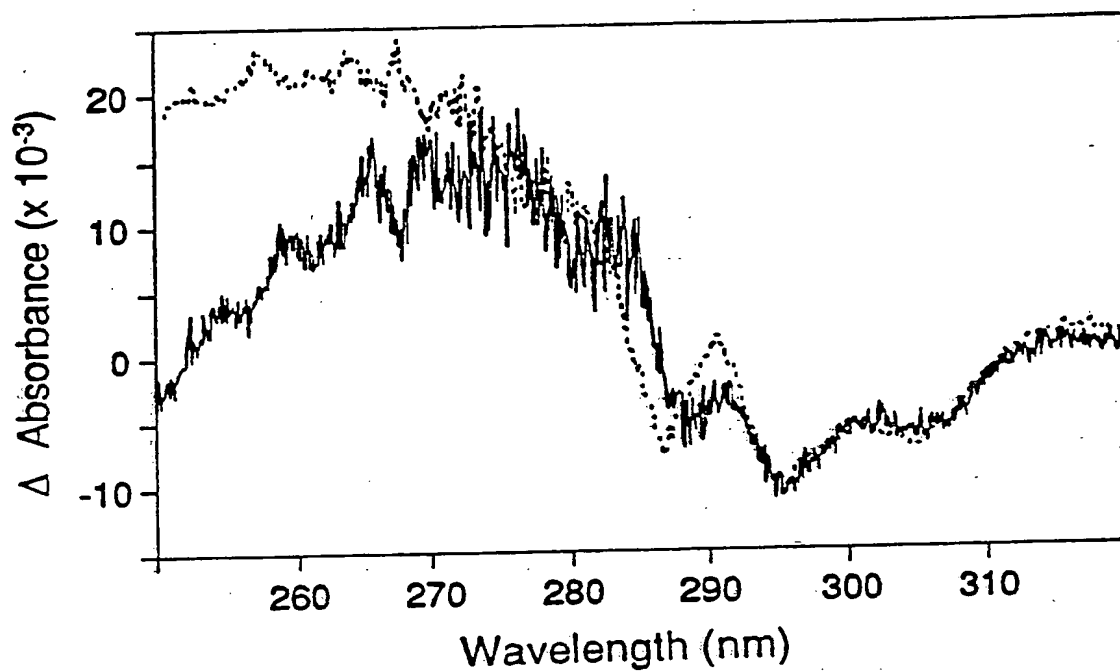
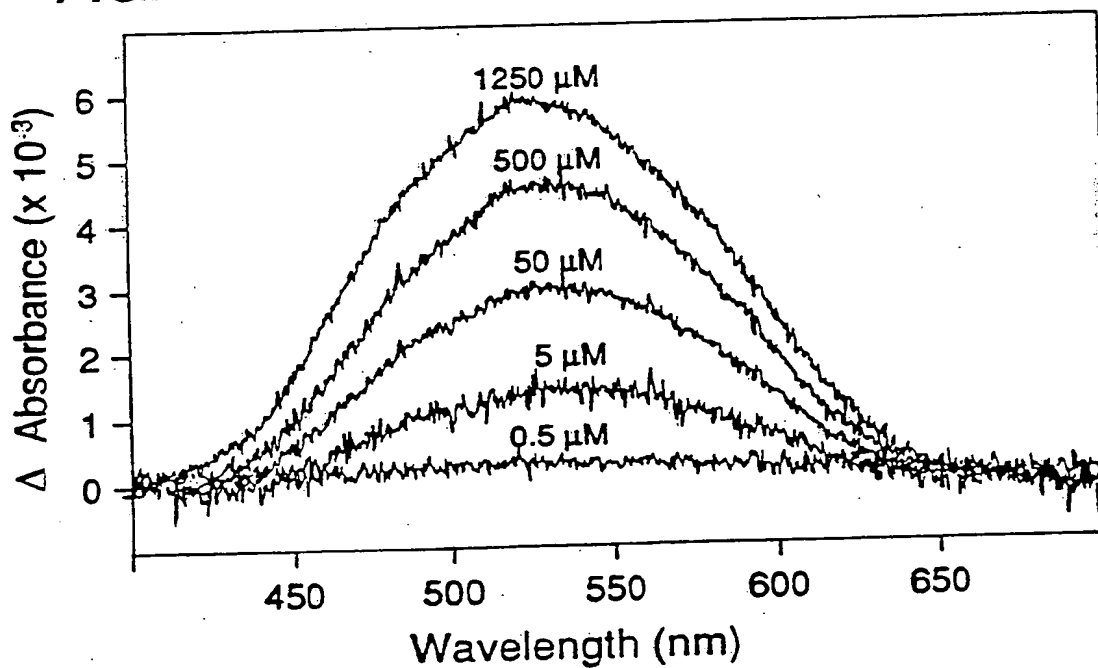


FIG. 3B



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**FIG. 4A****FIG. 4B**

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FIG. 5A

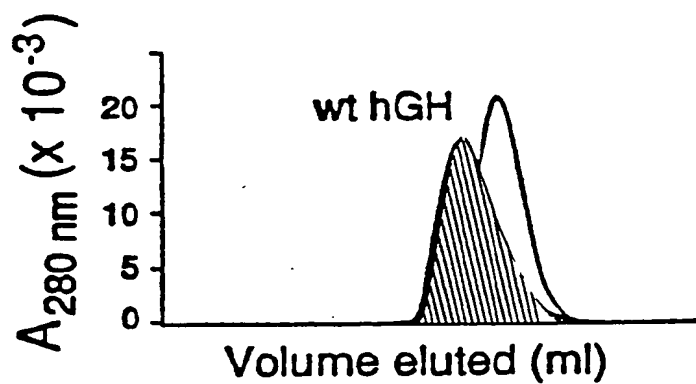


FIG. 5B

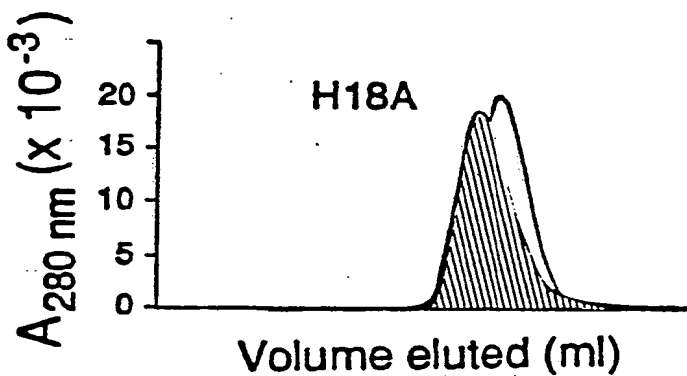


FIG. 5C

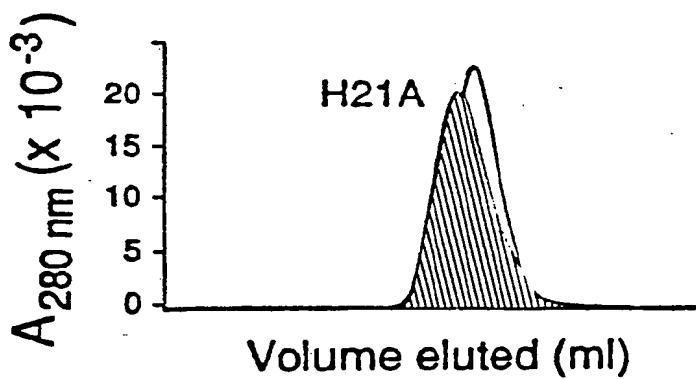
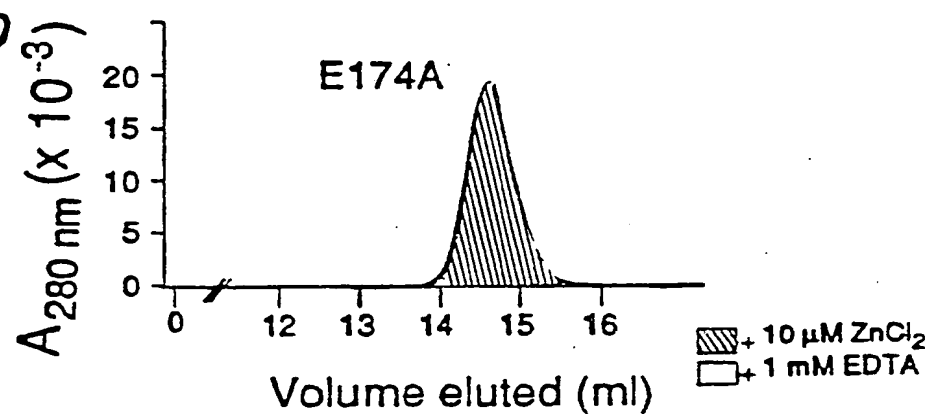


FIG. 5D



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FIG. 6A

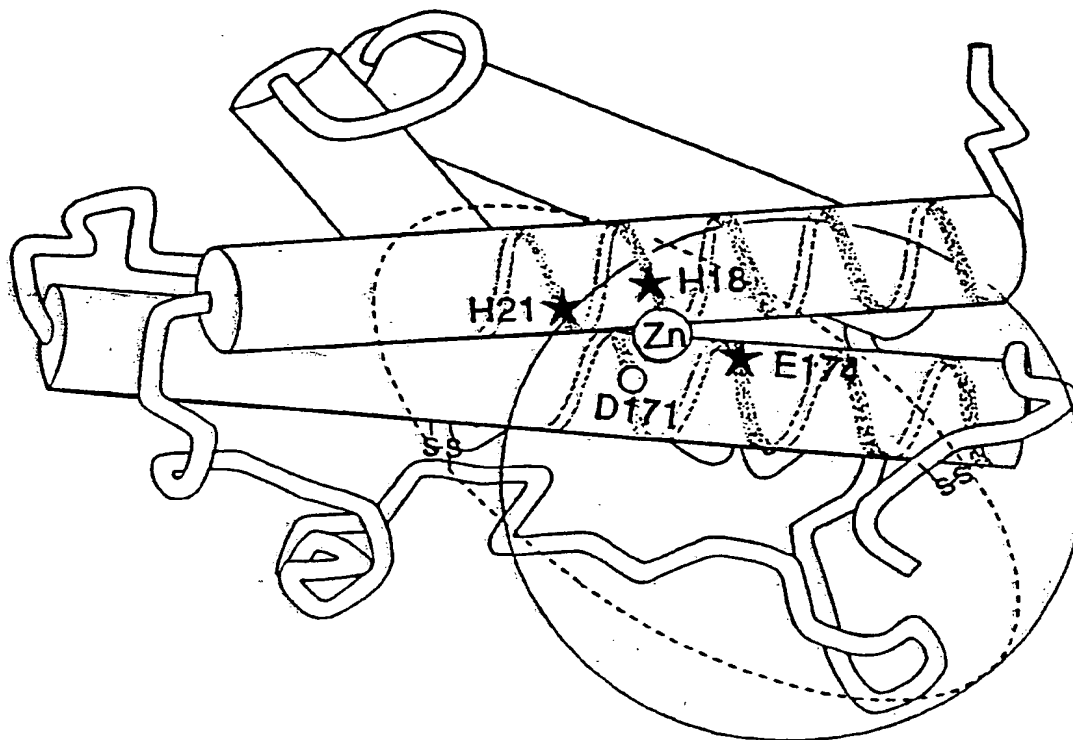
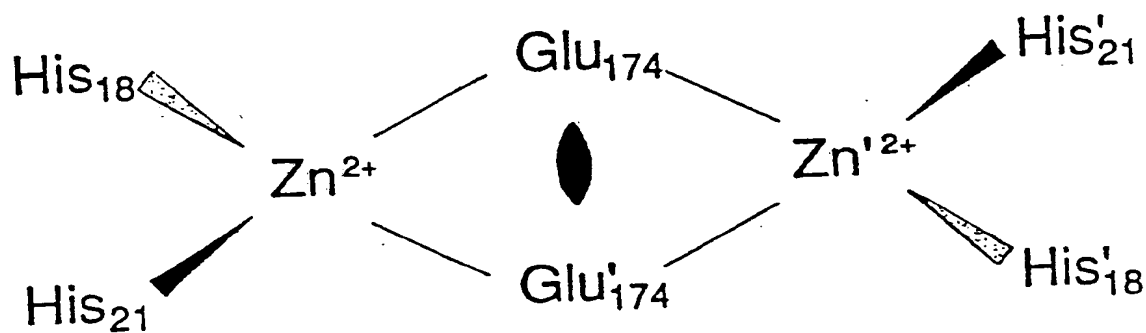


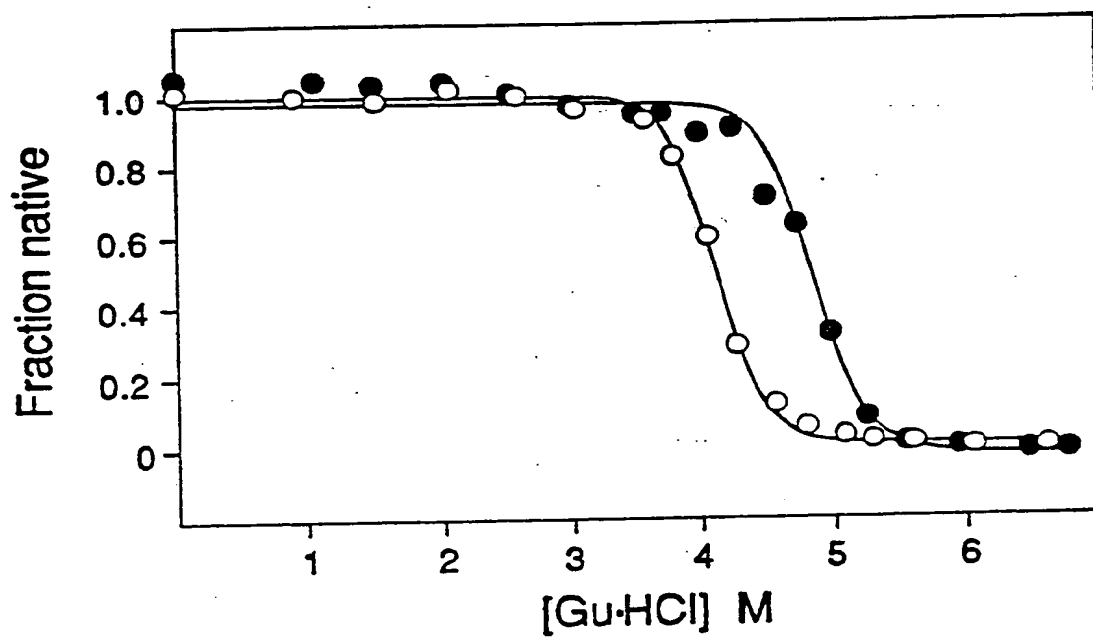
FIG. 6B



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FIG. 7



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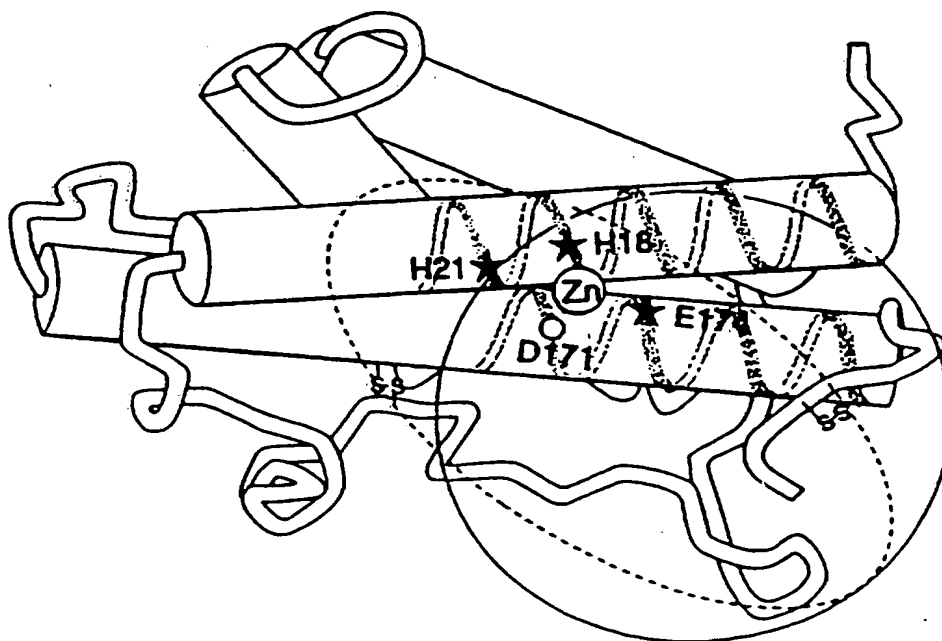
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> :  A61K 37/36	A3	(11) International Publication Number: WO 92/17200 (43) International Publication Date: 15 October 1992 (15.10.92)
<p>(21) International Application Number: PCT/US92/02365</p> <p>(22) International Filing Date: 25 March 1992 (25.03.92)</p> <p>(30) Priority data: 676,645 28 March 1991 (28.03.91) US</p> <p>(71) Applicant: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</p> <p>(72) Inventors: CUNNINGHAM, Brian, C. ; 24 Olive Avenue, Piedmont, CA 94611 (US). MULKERRIN, Michael, G. ; 2028 Arbor Avenue, Belmont, CA 94402 (US). WELLS, James, A. ; 1341 Columbus Avenue, Burlingame, CA 94010 (US).</p>	<p>(74) Agents: HENSLEY, Max, D. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 23 December 1992 (23.12.92)</p>	

(54) Title: STABLE GROWTH HORMONE METAL ION FORMULATIONS



(57) Abstract

Described are stable divalent metal ion-human growth hormone (hGH) complexes resulting in a stabilized hGH formulations through the formation of hGH-metal ion dimers. The stable dimers are characterized and therapeutic formulations of zinc and hGH are described.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/02365

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5                      A 61 K 37/36		
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched? Classification System                      Classification Symbols Int.Cl.5                      A 61 K Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	GB,A, 885798 (ORGANON LAB. LTD) 28 December 1961, see the whole document	1,2,4-6 ,8,9,12 ,20 21-24 13-19
A	---	
Y		
X	EP,A,0216485 (INTERNATIONAL MINERALS AND CHEMICAL CORPORATION) 1 April 1987, see page 2, lines 12-23; page 3, lines 14-28; page 4, line 33 - page 7, line 33; claims 1,2,4,5 (cited in the application)	1-6,8,9 ,12,20
A	---	
	---	21-24
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<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 04-08-1992		Date of Mailing of this International Search Report 27. 11. 92
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP,A,0343696 (MONSANTO COMPANY) 29 November 1989, see page 8, line 37 - page 9, line 53; claims 1-4,10,13 ---	1,2,12- 14
Y	WO,A,8909614 (GENENTECH, INC.) 19 October 1989, see page 6, line 20 - page 9, line 34 -----	13-19

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/ 02365

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 24,25 are directed to a method of treatment of  
(diagnostic method practised on) the human/animal body the search has been  
carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such  
an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- 1.- Claims 1-29
- 2.- Claims 30-31
- 3.- Claims 32-33

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-29

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9202365  
SA 59400

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP-A- 0216485	01-04-87	AU-B- 596112	26-04-90
		AU-A- 6109286	19-02-87
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		EP-A- 0409870	30-01-91
		JP-T- 3503764	22-08-91

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82